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USE OF TERPENES FOR THE TREATMENT OF DIGESTIVE TRACT INFECTIONS

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(57) Abstract: The prevention and treatment of digestive tract infections in humans and animals by orally administering a single terpene, a terpene mixture or a liposome-terpene (s) composition before or after the onset of a gastro-intestinal infection is described. Such infections may include traveller's diarrhea, ulcers, anthrax and other bacterial and parasitical infections.

USE OF TERPENES FOR THE TREATMENT OF DIGESTIVE TRACT INFECTIONS

The present invention relates to the treatment of microbial infections, especially the prevention and treatment of digestive tract infections in humans and animals, by orally administering a single terpene, a terpene mixture or a liposome-terpene(s) composition before or after the onset of the infection.

Digestive tract infections are mainly caused by pathogenic and opportunistic microorganisms and toxins produced by them. These illnesses are present in all types of animals and humans.

Diseases caused by organisms pathogenic to humans and animals are very common and encompass a range from the trivial to the lethal. With the arrival of the so-called 'antibiotic age' following World War II, it was hoped that the scourge of infection

would be I largely controlled on a permanent basis. However, this has not proved to be the case and in recent years many formerly useful prior art anti-bacterials have become ineffective as resistance has emerged. In the case of fungal infections the armamentarium has always been limited and the need remains for additional and more effective treatments.

In recent years, a number of particularly difficult problems have emerged and these have engaged considerable public concern. For instance, the rapidly rising prevalence of multiply resistant *Staphylococcus aureus* (MRSA) in hospitals in Western countries which has led to many deaths and, to all intents and purposes, only Vancomycin now stands as a fall-back treatment. Another example is outbreaks of severe *E. coli* infection, such as that in Scotland in the late nineteen-nineties which killed over 150 people. In the case of *E. coli*, there are particular problems in respect of treatment in that, even if the organism is killed quickly, the patient may die as the result of endotoxins being released from the organism if it is lysed as a result of anti-microbial attack.

Not all the mechanisms governing the emergence of resistance to anti-bacterials are understood but sufficient is known to suggest strongly that whilst a fairly simple game of molecular roulette will produce new anti-bacterials, any such product will

not remain free of resistance for long. Thus, it would appear that any solution to this apparently intractable problem of reduced effectiveness in anti-bacterials would need to be radically different to those employed in the prior art.

Recently with the scare of bio-terrorism there has been an increased concern with pathogens that can produce deadly outbreaks. This is the case with anthrax. Anthrax is considered a potential agent for use in biological warfare. Anthrax is an acute infectious disease caused by the spore-forming bacteria *Bacillus anthracis*. Anthrax is primarily a disease of domesticated and wild animals, particularly herbivorous animals. Humans become infected with anthrax by handling products from infected animals or by inhaling anthrax spores from contaminated animal products. Anthrax can also be spread by eating undercooked meat from infected animals. Anthrax infection can occur in three forms: cutaneous, inhalation, and gastrointestinal. The most common form is the cutaneous anthrax infection, which occurs when bacteria enter a cut or abrasion on the skin. This infection begins as a raised itchy bump that develops into a vesicle and then a painless ulcer, usually 1-3 cm in diameter, with a characteristic black necrotic area in the center. About 20% of untreated cases of cutaneous anthrax result in death. Deaths may be prevented with prompt antimicrobial treatment. The inhalation form has early symptom similar to a

common cold which progressively results in severe breathing problems. This type of anthrax is usually fatal. The intestinal form is characterized by an acute inflammation of the intestinal tract. The initial signs are nausea, loss of appetite, vomiting, and fever followed by abdominal pain, vomiting of blood and severe diarrhea. Intestinal anthrax results in death in 25% to 60% of cases. Anthrax is treated with antimicrobials and can be prevented with vaccination. The Department of Defense in the USA has a mandatory anthrax vaccination of all active military personnel.

Another digestive infection in humans is traveller's diarrhea, which affects over seven million visitors to high-risk tropical and semitropical areas every year. Others suggest that the incidence of traveller's diarrhea is 15 - 56% among international travelers. Approximately 1% of the sufferers are hospitalized, at least 20% are confined to bed for a day and nearly 40% have to change plans in their travel itinerary.

Traveler's diarrhea, defined as the passage of more than 3 unformed stools in a 24-hour period, is a self-limiting illness lasting 3 - 5 days. The illness may be presented either as (1) acute watery diarrhea (2) diarrhea with blood (dysentery) or (3) chronic diarrhea, often with clinical nutrient malabsorption.

Several factors contribute to the development of diarrhea in travelers, including personal (age, socioeconomic status, body weight, preexisting gastrointestinal illnesses), behavioral (mode of travel, standard of accommodation, eating in public places, dietary errors) and travel related (destination, duration of stay, country of origin, season). Approximately 85% of lithe diarrheas among international travelers are produced by bacterial enteropathogens. These pathogens are usually acquired through ingestion of fecally contaminated food or water. Sometimes dirty hands or insects are the vectors of fecal contamination. Cooked food is safe to consume as long as the temperature at the interior of the food reaches 160°F or more. An undercooked hamburger is risky food, because ground meat can become contaminated at the processing plant and during preparation.

The common pathogens that produce traveler's diarrhea include *Clostridium difficile, Yersenia enterolitica, Shigella sp., Campylobacter sp., Salmonella sp.*, ETEC (enterotoxigenic) and EAEC (enteroaggregative) *Escherichia coli*. Traveler's diarrhea produced by *Shigella sp.* or *Salmonella sp.* tend to cause a more severe and longer lasting disease than that caused by the most common cause, enterotoxigenic *E. coli* (ETEC). *Campylobacter jejuni is* a relativelly common cause of traveler's diarrhea especially in the winter. Viruses such as

rotavirus, cytomegalovirus and Norwalk agent are less common causes.

There are several groups of pathogenic *E. coli*. They include Enterotoxigenic (ETEC), which produce a range of toxins, heat-stable or heat-labile in nature. ETEC is the most common cause of diarrhoea disease in children in the developing world; it also causes many travelers' diarrhoea cases.

Verocytotoxic *E. coli* (VTEC) strains produce toxins that destroy the gut mucosa and can cause kidney damage; *E. coli* 0157 H:7 is the most publicised example of this type.

Enteropathogenic *E. coli* (EPEC) do not appear to produce toxins but may attach the microvilli, this group often causes infection in babies and young children.

Enteroinvasive *E. coli* (EIEC) attaches to the mucosal lining of the large intestine and invade the cells, causing tissue destruction and inflammation. EIEC are usually food borne pathogens and are an important cause of disease in areas of poor hygiene.

The severity of the disease symptoms are dependent on the strain encountered and the underlying health of the individual. EIEC and VTEC strains can cause very serious disease (haemorrhagic colitis and

renal failure) and require hospitalisation. Milder cases are usually treated by fluid and electrolyte replacement and rest.

The use of antibiotics limits the course of diarrhea to a little over a day compared with an average of over 3 - 5 days when diarrhea remains untreated. The widespread resistance of the traditional antimicrobial agent, Trimethoprim plus sulfamethoxazole (TMP/SMX), and fluoroquinolones are the main reasons of concern about the continuous use of antimicrobials for the treatment of traveler's diarrhea (Dupont et al, 1998). The extensive use of antibiotics can also lead to overgrowth syndromes, *Candida vaginitis* can occur, the overgrowth of *Clostridium difficile* due to less competitive environment in the gastrointestinal tract can also result in diarrhea.

Short-term travelers that have experience diarrhea do not develop protection, since it requires continued exposure to enteropathogens to develop immunological protection against traveler's diarrhea. Vaccination is a promising option, but vaccines against all enteropathogens that cause traveler's diarrhea have not been developed. Other protection methods to treat traveler's diarrhea are: the use of nonabsorbed antimicrobials, which have fewer side effects and should be safer to use in children and pregnant women in whom quinolones are contraindicated; antisecretory and antimotility

agent (loperamide); the use of attapulgite, a hydrated aluminum silicate clay preparation; and probiotics i.e. lactobacillus, which appear to be useful in the prevention or treatment of travelers diarrhea. In all cases the restoration of water and electrolyte balance is necessary. The following table shows the current treatments for Traveler's Diarrhea:

Agent	Efficacy	Comments
Activated charcoal	Not efficacious	May absorb important medications
Lactobacillus	Not proven	Safe
Bismuth subsalicylate preparations	65% protective	Rinse mouth to avoid black tongue
Trimethropim-Sulfamethoxazole	70-80% protective	Resistance rising worldwide
Fluoroquinolones (norfloxacin, ciprofloxacin, Ofloxacin)	90% protective or better	Currently most effective antimicrobial but resistance rising worldwide.

Ericsson, Charles (1998)

In humans and animals, peptic ulcers are open sores produced by a bacteria. These open sores can be present on the entire gastro-intestinal tract, mainly esophagus, stomach and proximal part of the small intestine. There is evidence that support the role of *H. pylori* as the etiologic agent of chronic gastritis and peptic ulcer. *H. pylori*, a

gram-negative, microaerophilic spiral bacteria is the major cause of gastro-duodenal disease, including chronic gastritis, gastric and duodenal ulcers and gastric neoplasia. Greater than 50% of North American adults over 50 years of age are infected with *H. pylori*. In contrast, in some developing and newly industrialized countries virtually all adults are infected. In developing countries almost all children are infected by age 10, whereas in developed countries only the children of lower socioeconomic levels are infected. H. *pylori* is characterized by very high urease activity that may be associated with virulence, in the absence of urea *H. pylori* is sensitive to acidic pH. Urease activity may be an important colonization and survival factor by generating ammonia in the immediate bacterial microenvironment. *H. pylori* has been classified as a type 1 carcinogen by the World Health Organization because of the danger of persistent infection with the bacterium causing gastric cancer. *H. pylori* infection is of extreme importance in the causation of peptic ulcer disease. By initiating a gastritis or dyspeptic symptoms, it can predispose to subsequent episode of either gastric lymphoma or stomach cancer.

The eradication of *H. pylori* has been obtained with combination therapy, triple therapy using bismuth plus two antibiotics (metronidazole and either amoxicillin or tetracycline has been effective).

Problems due to development of antimicrobial resistant and side effects (diarrhea, nausea, abdominal pain and others) may explain why the use of antibiotics has not become a preferred treatment for gastritis and peptic ulcers due to *H. pylori*.

Antibacterial treatment of *H. pylori* is difficult because of the habitat occupied by the organism below the layer of the mucus adherent to the gastric mucosa. Access of antibacterial agents to this site is limited from the lumen of the stomach and also from the gastric blood supply.

The use of medium chain fatty acids and medium chain triglycerides has been shown to inhibit the growth of H. pylori in vitro. The mechanism by which they exert antibacterial effect is thought to involve: 1) damage to the bacterial outer membrane leading the increase membrane fluidity and permeability, 2) Incorporation of these fatty acids, making the bacterial membrane unstable, 3) Production of peroxides due to oxidation of fatty acids.

The mode of transmission of *H. pylori* in humans is still poorly understood. There are reports of detection of this microorganism in the oral cavity and in the feces. If *H. pylori* is harvested in the oral cavity or bowel, these might represent important reservoir for the reinfection and transmission with consequences from treatment. One

vector for the transmission of *H. pylori* are flies, they can carry viable *H. pylori* in their external surfaces and alimentary tracts.

In animals, the presence of scours in calves is of economic importance. It is estimated that the death lost of calves less than 6 months of age is approximately 2.5% or over 100,000 a year. Most of the mortality and morbidity of the calves are due to infectious diseases, mainly scours. More than 90% of scours in calves is produced by *E. coli* and *Salmonella*. Clostridia has proved to be fatal in the majority of cases. There are preventive methods like (I) vaccination of the mothers in order to passively transfer antibodies in colostrum; (2) the use of immunological supplements for milk replacers; (3) the use of probiotics to create a gastro-intestinal healthy environment (4) changes in calf management. None of these protective measures are 100% effective.

Another animal of economic importance is swine. The incidence of diarrhea in neonates and weaned piglets is very high. Again, *E. coli* and *Salmonella* are the main microorganisms involved in diarrhea in swine. There are losses in the nursery while piglets are still lactating and after weaning. There are similar preventive methods as in calves. One of the preferred methods is segregated early weaning (SEW). The basis of early weaning is that the earlier piglets are weaned from

the sow the less are the chances of crossover diseases between sow and piglets. This method requires the use of antibiotics.

In both cases, calf and piglet scours, the preferred method of treatment is antibiotics. The European Community has banned the use of 5 antibiotics and in the Unites States the FDA is banning the use of fluoroquinolone in animals due to the development of *Campylobacter* resistant to this antibiotic. Bacteria resistance has encouraged the development of antibiotic-alternative products.

Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Their building block is the hydrocarbon isoprene $(C_5H_8)_n$. Terpenes have been found to be effective and nontoxic dietary antitumor agents which act through a variety of mechanisms of action (Crowell and Gould, 1994 and Crowell et al, 1996). Terpenes, i.e. geraniol, tocotrienol, perillyl alcohol, b-ionone and d-limonene, suppress hepatic HMG-COA reductase activity, a rate limiting step in cholesterol synthesis, and modestly lower cholesterol levels in animals (Elson arid Yu, 1994). D-limonene and geraniol reduced mammary tumors (Elegbede et al, 1984 and 1986 and Karlson et al, 1996) and suppressed the growth of transplanted tumors (Yu et al, 1995).

Terpenes have also been found to inhibit the in-vitro growth of bacteria and fungi (Chaumont and Leger, 1992, Moleyar and Narasimham, 1992 and Pattnaik, et al, 1997) and some internal and external parasites (Hooser, et al, 1986). Geraniol was found to inhibit growth *of Candida albicans* and *Saccharomyces cerevisiae* strains by enhancing the rate of potassium leakage and disrupting membrane fluidity (Bard, et al, 1988). B-ionone has antifungal activity which was determined by inhibition *of* spore germination, and growth inhibition in agar (Mikhlin et al, 1983 and Salt et al, 1986). Teprenone (geranylgeranylacetone) has an antibacterial effect on *H. pylori* (Ishii, 1993). Solutions of 11 different terpenes were effective in inhibiting the growth of pathogenic bacteria in in-vitro tests; levels ranging between 100 ppm and 1000 ppm were effective. The terpenes were diluted in water with 1% polysorbate 20 (Kim et al, 1995). Diterpenes, i.e. trichorabdal A (from R. Trichocarpa) has shown a very strong antibacterial effect against *H. pylori* (Kadota, et al, 1997).

Rosanol a commercial product with 1% rose oil has been shown to inhibit the growth of several bacteria *(Pseudomona, Staphylococus, E. coli and* Hpylori). Geraniol is the active component (75%) of rose oil. Rose oil and geraniol at a concentration of 2 mg/litre inhibited the growth of *H pylori* in vitro. Some extracts from herbal medicines have been shown to have an inhibitory

effect on *H. pylori*, the most effective being decursinol angelate, decursin, magnolol, berberine, cinnamic acid, decursinol and gallic acid (Bae, et al 1998). Extracts from cashew apple, anacardic acid and (E)-2-hexenal, have shown bactericidal effect against *H. pylori*.

There may be different modes of action *of* terpenes against *H. pylori*. They could (1) interfere with the phospholipid bilayer of the cell membrane (2) impair a variety of enzyme systems (HMG-reductase) and (3) destroy or inactivate genetic material.

SUMMARY OF THE INVENTION

Prevention and treatment of digestive tract infections by orally administering a biocidal terpene, a biocidal terpene mixture or a liposome-terpene(s) composition before of after the onset *of* the infection.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Digestive tract infections not only are an uncomfortable illness for humans but also are of economic importance for the animal industry. In some cases the illness can cause death in children, elderly and immune-compromised people. The preferred treatment of the disease is antibiotics. The extensive use of antibiotics in humans and the animal industry has created the development of antibiotic-resistant bacteria. The increased

antibiotic resistance has beer the main reason to seek new antimicrobial alternatives. The European Community has banned the use of 5 antibiotics in animals and in the Unites States the FDA is banning the use of fluoroquinolone in animals due to the development of *Campylobacter* resistant to this antibiotic.

Terpenes, which are GRAS (Generally Recognized As Safe) have been found to inhibit the growth of cancerous cells, decrease tumor size, decrease cholesterol levels and have a biocidal effect on microorganisms in vitro. Onawunmi (1989) showed that growth media with more than 0.01 % citral reduced the concentration of E. coli and at 0.08% there was a bactericidal effect. Barranx, et al (1998) teach us a terpene formulation, based on pine oil, used as a disinfectant or antiseptic cleaner. Koga, et al (1998) teach that a terpene found in rice has antifungal activity. Iyer, et al (1999) teach us an oral hygiene antimicrobial product with a combination of 2 or 3 terpenes that showed a synergistic effect. Neither of them suggested the use of a terpene, terpene mixture or liposome-terpene(s) combination for the prevention or treatment of gastro-intestinal infections i.e. traveler's diarrhea.

Several US Patents (US#5,547,677, US#5,549,901, US#5,618,840, US#5,629,021, US#5,662,957, US#5,700,679, US#5,730,989) teach us that certain

types of oil-in-water emulsions have antimicrobial, adjuvant and delivery properties.

Thus, the present invention provides a composition for preventing or treating gastro-intestinal infections, wherein said composition comprises a terpene or a mixture of terpenes. We have found that certain mixture of terpenes are synergistically effective, relative to the effects of the component terpenes administered separately. Thus terpenes having biocidal activity which in combination with two or more other terpenes synergistically increase the biocidal effectiveness are of especial interest.

One composition of interest comprises a mixture of carvone and geraniol, optionally together with other terpenes. The content of carvone and geraniol may each be from 10 to 90% (by weight), but is preferably 10 to 60% by weight. Other terpenes which may be present include citral, b-ionone, eugenol, terpeniol, carvacrol, anethole or the like. These optional additional terpenes may be present at 5 to 50% by weight, for example 10 to 40% by weight.

Optionally, the terpenes may be presented in the form of liposomes.

Liposomes are microscopic structures consisting of concentric lipid bilayers enclosing an aqueous

space. Liposomes are classically prepared from phospholipids which occur naturally in animal cell membranes, but several synthetic formulations are now commonly used. The lipid composition of the liposome can be varied to give liposomes different physical characteristics i.e. size and stability. Liposomes can be prepared by the reverse-phase evaporation or dehydration-rehydration vesicle methods using a mixture of dipalmitoyl phosphatidyl choline, cholesterol, dipalmitoyl phosphatidyl glycerol, dipalmitoyl phosphatidyl ethanolamine and other synthetic fatty acids and emulsifiers. When making liposomes first multilamellar vesicles are formed spontaneously when amphipathic lipids are hydrated in an aqueous medium. Unilamellar vesicles are often produced from multilamellar vesicles by the application of ultrasonic waves.

Multilamellar vesicles can be prepared by the procedure known as dehydration-rehydration. Briefly, egg phosphatidylcholine and cholesterol are mixed in chloroform, dried in a rotary evaporator, dilute with water and sonificated to form unilamellar vesicles. The solution is freeze dried and rehydrated with the terpene solution in order to embed the terpene inside the liposome. Another method to produce liposomes is by mixing together lipids, an emulsifier and the terpenes. The emulsion is obtained by using a Polytron homogenizer with special flat rotor that creates an emulsion. The lipids could consist of soybean oil,

any commercial or pharmaceutical oil; the emulsifier consist of egg yolk lecithin, plant sterols or synthetic including polysorbate-80, polysorbate-20, polysorbate-40, polysorbate-60, polyglyceryl esters, polyglyceryl monooleate, decaglyceryl monocaprylate, propylene glycol dicaprilate and triglycerol monostearate. The lipid concentration in the oil phase is 75-95% and the emulsifier concentration from 5-25%. When preparing the emulsion a ratio oil to water could vary from 10-15 parts lipid to 35-40 parts terpenes diluted in water at a concentration of 0.5% to 50%. Once the emulsion is formed this is combined with a carrier in order to be use as a humectant, cream or other suitable carrier for topical applications. The emulsion concentration use for topical application varies from 0.0055 through 1.0% of the final product. Several modifications to the emulsion can be achieved by simply varying the concentration and type of terpenes used. This modification can give us different products with different antimicrobial specificity.

By encapsulating terpenes within these emulsions the antimicrobial effect will be increased: (1) the liposome will disrupt the bacterial membrane and (2) the terpenes will be more effective in disrupting cytoplasmatic enzymes.

It will be apparent for those skilled in the art that the aforementioned objects and other

advantages may be further achieved by the practice of the present invention.

EXAMPLE 1: Preparation of the terpene mixture

The terpene, terpene mixture or liposome-terpene(s) combination consists of a blend of generally recognized as safe (GRAS) terpenes with a GRAS surfactant. The ratio of terpenes is from 1-99% and the surfactant ratio from 1-99% of the mixture. The terpenes, comprised of natural or synthetic terpenes, are citral, b-ionone, geraniol, eugenol, carvone, terpeniol, carvacrol, anethole or other terpenes with similar properties. The surfactant is preferably polysorbate-80 or other suitable GRAS surfactants.

EXAMPLE 2: Preparation of liposomes containing terpenes

Any standard method for the preparation of liposomes can be followed with the knowledge that the lipids used are all food-grade or pharmaceutical-grade. A set amount of lipids, an emulsifier and the terpenes was used to prepare an emulsion. The emulsion was obtained by using a Polytron homogenizer with special flat rotor that created an emulsion. The lipids consisted of soybean oil, any commercial or pharmaceutical oil; the emulsifier consist of egg yolk lecithin, plant sterols or synthetic emulsifiers including

polysorbate-80, polysorbate-20, polysorbate-40, polysorbate-60, polyglyceryl esters, polyglyceryl monooleate, decaglyceryl monocaprylate, propylene glycol dicaprilate and triglycerol monostearate. A solution containing 75-95% lipids (oil) and 5-25% emulsifier consisted of the oil phase. The aqueous phase consisted of the terpene diluted in water at a rate of 0.5% to 50%. To form the emulsion a ratio of oil to water varying from 10-15 parts lipid (oil phase) to 35-40 parts terpenes (aqueous phase) was mixed. Any standard method for the preparation of liposomes can be followed with the knowledge that the lipids used are all food-grade or pharmaceutical-grade. The suspension containing a lipid, an emulsifier and the terpenes is emulsified with a Posytron homogenizer until a complete milky solution is obtained.

EXAMPLE 3: Preparation of liposomes

This step consists of the preparation of the terpene(s)-liposome combination by mixing 99% of liposome and 1% of terpene mixture. Several combinations of this formulation can be obtained by varying the amount of terpene and liposome from 1% to 99%. The liposomes are prepared as in Example 2 without the addition of terpenes in the formulation.

EXAMPLE 4: In-vitro effectiveness of terpenes against *E. coli*

This example demonstrates the effect of terpenes on the cell membrane fragility of *E. coli*, which is considered indicative of other pathogenic bacteria such as *Salmonella* and *Listeria*. Lysis of the cell membrane was monitored by the determination of galactosidase activity. β-galactosidase is a well-characterized cytosolic enzyme in bacteria. This enzyme is inducible in the presence of isopropyl-1-thiogalactosidase (IPTG) and assayed colorimetricaly with substrate o-nitro-phenyl-β-D-galactoside (ONPG). ONPG is cleaved to release o-nitrophenol with peak absorbance at 420 nm. Since intact *E. coli* is impermeable to both ONPG and the enzyme, the cells have to be lysed prior to enzymatic assay. Therefore the ability of terpenes to lyse *E. coli* can be measured with this enzymatic assay and compared to known lysing agents.

The procedure used was as follows: *E. coli* strains AW574 or AW405 were cultured overnight in 10 ml tryptone broth with 1 nM IPTG at 35°C. Cells were allowed to grow until an absorbance equal to 0.9 was reached. Cells were harvested, washed with phosphate buffer and resuspended to an absorbance equal to 0.5. 0.1 ml of the bacteria culture was added to 0.9 ml of buffer, warmed to 30°C and then 80 µl of terpenes (85% terpenes and 15% polysorbate-80), 80 µl water (background) or 40 µl chloroform plus 40 µl 1% SDS in water (positive

control) were added. After the addition of the lysing agents the tubes were mixed for 10 seconds and 0.2 ml of ONPG (4 mg/ml water) was added, then incubated for 5 minutes. The enzyme activity was stopped with 0.5 ml of 1 M sodium carbonate. After being centrifuged for 3 minutes at 1,500 x g, supernatant was transferred to cuvettes and read at 420 nm. The relative degree of lysis caused by terpenes was calculated as follows:

100 x (OD terpenes-OD water) / (OD chloroform-OD water)

This shows that dosages can be manipulated to either lyse the cell outright, or in the case of lower dosages, stop bacterial growth without lysis of the cell membrane. The advantage of this controllable result is the ability to prevent lysis and the resultant release of endotoxins where contraindicated.

Table 1: Lysis of *E. coli* by Terpenes

Terpenes (μM)		Relative lysis %
Carvone	404,000	NM*
	40,400	54
	4,040	22
	404	3.2
Geraniol	363,000	NM
	36,300	96
	3,630	98
	363	34
	36.3	4
	3.63	2.4
b-Ionone	308,000	NM
	30,800	NM
	3,080	NM
	308	52
	30.8	44
	3.08	23
	0.308	4.78
	0.0308	1.3
80 μl Polysorbate-80		3.2
80 μl Polysorbate-80 + SDS + Chloroform		100
SDS + Chloroform		100*

*Lysis due to chloroform and SDS combination was considered to be 100%.

*NM, not measurable due to formation of turbid colloidal solution.

EXAMPLE 5: In in-vitro effectiveness of terpenes against several microorganisms

This example demonstrate the effectiveness of terpenes against *Escherichia coli*, *Salmonella typhimurium*, *Pasteurella mirabilis*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillius fumigates*. Each organism, except A. *fumigatus*, was grown overnight at 35-37°C in tryptose broth. *A. fumigates* was grown for 48 hours. Each organism was adjusted to approximately 10^5 organisms/ml with sterile saline. For the broth dilution test, terpenes were diluted in sterile tryptose broth to give the following dilutions: 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16,000, 1:32,000, 1:64,000 and 1:128,000.

Each dilution was added to sterile tubes in 5 ml amounts. Three replicates of each series of dilutions were used for each test organism. 0.5 ml of the test organism was added to each series and incubated at 35-37°C for 18-24 hours. After incubation the tubes were observed for growth and plated onto blood agar. The tubes were incubated an additional 24 hours and observed again. The A. *fumigates* test series was incubated for 72 hours. The minimum inhibitory concentration for each test organism was determined as the highest dilution that completely inhibits the organism.

Table 2: Results of the inhibitory activity of different dilutions

Organism	Visual assessment of growth *			Growth after subculture to agar plates *			Mean inhibitory dilution
	1	2	3	1	2	3	
S. typhimurium	500	500	500	500	500	500	500
E.coli	1000	1000	1000	1000	1000	1000	1000
P. mirabilis	1000	1000	1000	1000	1000	1000	1000
S.aureus	1000	1000	1000	1000	1000	1000	1000
C albicans	1000	1000	1000	1000	1000	1000	1000
A. fumigatus	8000	16000	16000	8000	16000	16000	13300

* The results of the triplicate tests with each organism as the reciprocal of the dilution that showed inhibition/killing

** NI = not inhibited

EXAMPLE 6: In in-vitro effectiveness of terpenes against *Escherichia coli* over time.

This example demonstrates the effectiveness of terpenes at several concentrations against *Escherichia coli* and cultured over time. Terpene dilutions (1:500, 1:1000, 1:2000, 1:4000, 1:8000, and 1:16,000) were prepared in BHI broth and in saline. These were prepared in 25 ml amounts. *E. coli* was grown overnight in BHI broth and diluted to a MacFarland 0.5 concentration in saline. This solution was diluted 1:100 to be used to inoculate

(0.5 ml) each terpene dilution tube. The series that contained the terpene dilution in BHI was tested at 30 min, 90 min, 150 min and 450 min. Each tube was mixed and serially diluted in saline. 0.5 ml of each dilution was spread plated onto MacConkey (MAC) agar plates. Also, 3 drops of the undiluted and the 1:100 dilution was added into respective tubes of BHI broth. The tubes and plates were incubated overnight at 35°C. The series that contained the terpene's dilution in saline were tested at 60 min, 120 min, 180 min and 480 min. Each tube was mixed and serially diluted in saline. 0.5 ml of each dilution was spread plated onto MacConkey (MAC) agar plates. Also, 3 drops of the undiluted and the 1:100 dilution were added into respective tubes of BHI broth. The tubes and plates were incubated overnight at 35°C.

Table 3: Subculture from the tubes containing various dilutions of terpenes in broth

Time	Dilution	1:500	1:1000	1:2000	1:4000	1:8000	1:16,000
30 min	Undiluted	NG	+	+	+	+	+
	1:100	NG	+	+	+	+	+
90 min	Undiluted	NG	NG	+	+	+	+
	1:100	NG	NG	NG	+	+	+
150 min	Undiluted	NG	NG	+	+	+	+
	1:100	NG	NG	NG	+	+	+
450 min	Undiluted	NG	NG	+	+	+	+
	1:100	NG	NG	+	+	+	+

NG: no growth, +: growth

Table 4: Subculture from the tubes containing various dilutions of terpenes in saline

Time	Dilution	1:500	1:1000	1:2000	1:4000	1:8000	Control
60 min	Undiluted	NG	+	+	+	+	+
	1:100	NG	NG	NG	+	+	+
120 min	Undiluted	NG	NG	NG	+	+	+
	1:100	NG	NG	NG	NG	+	+
180 min	Undiluted	NG	NG	NG	+	+	+
	1:100	NG	NG	NG	NG	+	+
480 min	Undiluted	NG	NG	NG	NG	+	+
	1:100	NG	NG	NG	NG	NG	+

NG: no growth, +: growth

Table 5: The quantitative results of the activity of various terpene dilutions against *E.coli* (cfu)

Media	Time	1:500	1:1000	1:2000	1:4000	1:8000	Control
Broth	30 min	0	0	660	3600	3600	4600
	90 min	0	0	12	4600	5400	7600
	150 min	0	0	10	8000	12,000	14,000
	450 min	0	0	15,000	28×10^3	23×10^7	16×10^8
Saline	60 min	0	4	140	4000	2000	1300
	120 min	0	0	0	90	3800	2600
	180 min	0	0	0	2	2000	5000
	480 min	0	0	0	0	104	8000

NG: no growth, +: growth

EXAMPLE 7: In vitro effectiveness of selected terpenes on *Helicobacter pylori*.

This example shows the bactericidal effect of selected terpenes on the viability of *H. pylori*. Five terpenes (anethole, carvone, citral, geraniol and b-ionone) were used for this study. Terpenes were mixed to a ratio of 90% terpene plus 10% polysorbate-80. The *H. pylori*, used was strain #26695 of porcine origin, this bacteria is a motile, cag A, vac A cytotoxin-positive gram negative bacteria which colonizes gnotobiotic piglets and indefinitely persists within the gastric microenvironment as a superficial infection of the gastric mucosa and mucus layer.

The study was as follows:

1) Stock solutions of each terpene with polysorbate-80 were prepared (1.8 ml terpene plus 0.2 ml polysorbate-80).

2) Stock solutions were diluted in Brucella broth 10% (v/v) fetal calf serum to a final concentration of stock at 1:10, 1:50, 1:100, 1:500, 1:1000, 1:5000 and 1:10000. Controls consisted of 10% (v/v) polysorbate-80 in Brucella broth, Brucella broth alone and bacteria in Brucella broth.

3) A total of 1.0×10^6 bacteria (30 µl) was added to 970 µl terpene dilutions (final volume of 1.0

ml) in loosely capped tubes and incubated for 24 hours at 37 °C with continuous mixing.

4) Duplicate samples (0.1 ml) from each test dilution was titrated onto blood agar plates and incubated for 48 hours at 37°C on 10% CO_2 environment. Bacterial colony forming units (cfu) were determined by visual (counting) inspection. Recovered bacteria were confirmed to be *H. pylori* by catalase and urease enzyme activities.

The results are summarized in the following table:

Table 6: Effect of different terpenes on *H. pylori* growth

	Final dilution tested for antimicrobial effects against 10^6 cfu							
Terpene	1:10	1:50	1:100	1:500	1:1000	1:5000	1:10000	1:50000
Polysorbate -80	NG*	NG	NG	10^3	10^4	TNTC**	TNTC	TNTC
Anethole	NG	NG	NG	NG	10^3	10^3	TNTC	TNTC
Carvone	NG	NG	NG	NG	NG	10^4	TNTC	TNTC
Geraniol	NG	NG	NG	NG	NG	NG	10^2	TNTC
Citral	NG	NG	NG	NG	NG	NG	NG	TNTC
b-ionone	NG	NG	NG	NG	NG	NG	NG	TNTC

* NG = no growth ** TNTC = Too Numerous To Count

EXAMPLE 8: In vitro effectiveness of single or combination of terpenes against *E. coli*.

The objective of this example was to determine an optimum terpene mixture which could have a greater

biocidal effect. *E. coli* strain AW574 was grown in tryptone broth to an exponential growth phase (O.D. between 0.4 and 1.0 at 590 nm). One tenth of this growth was inoculated to 10 ml of tryptone broth followed by the addition of individual terpenes as indicated in Example 5; then incubated for 24 hours at 35-37°C and the O.D. determined in each tube. The concentration of terpenes was 1 or 2 µMol. Each treatment was repeated in triplicate. The results are expressed as percentage bacterial growth as compared to the control treatment. It is observed that the combination of terpenes give better biocidal effect than single terpenes, with geraniol and carvone better than b-ionone.

Table 7: Effect of single terpene or their combination against on *E. coli* growth

µMol terpenes			% Growth
B-ionone	Carvone	Geraniol	
0	0	0	100.00
2	0	0	84.00
0	2	0	63.00
0	0	2	54.00
1	1	1	41.00
1	2	1	31.10
1	1	2	14.80
1	2	2	15.90
2	1	1	48.60
2	2	1	44.30
2	1	2	30.20
2	2	2	1.50

EXAMPLE 9: In vitro effectiveness of a combination of terpenes against different *E. Coli* strains

Both well-test and broth test methods were used to assess the effect of terpene formulations against a variety of strains of *E. coli*. The broth test method was judged to be a more applicable simulation of gastrointestinal tract conditions than the well plate (zone of inhibition) method. A series of broth tests was conducted on a selected test formulation to determine its activity in an aqueous environment.

Test micro-organisms

Bacteria were sub-cultured from original American Type Culture Collection (ATCC) freeze-dried material. They included *E. coli* strains 8739, 25922 and 700728 (Serotype group O: 157 H:7), which are BioSafety class 1 organisms and *E.* coli 12795 (Serotype group O: 26) which is a BioSafety class 2 organism. All the bacteria were cultured on Tryptone Soya Agar (TSA), supplied by Oxoid Ltd, Hampshire and Mueller Hinton Agar (MHA), supplied by Merck Ltd. The incubation temperature was 35 °C.

Broth Test Procedure

E. coli cultures were prepared in nutrient broth and allowed to grow until exponential growth phase was achieved (16 hours at 35 °C). 1 ml of this culture was transferred to each of a series of pre-sterilised Duran bottles containing 100 ml nutrient broth, 0.5 % w/v Polysorbate 80 and this gave an initial inoculum of approximately 10^8 microbial cells per ml of broth.

The Duran bottles were agitated on a vortex shaker to produce good mixing and the Optical Density (OD) at 590 nm read on a calibrated Unicam UV 300 spectrophotometer controlled by Vision 32 software. The OD of a sample of a placebo broth was also recorded.

The bottles were then placed in an incubator at 35°C. The bottles were removed at 30 minute intervals and placed on a vortex shaker at level three for 30 seconds. The bottles were then returned to the incubator. The OD was recorded at hourly intervals, for up to 24 hours.

After completion of the tests, the broths were autoclaved on programme 4 of an AVX240 autoclave (132 °C for 30 minutes) to sterilise them.

The terpenoids tested in this series of exemplary experiments included I-carvone, citral and geraniol in varying proportions. One exemplary formulation, constituting the test formulation, is given in Table 8, below

Table 8	% w/w
I-Carvone	40
Citral	40
Geraniol	15
Polysorbate 80	5
Total	100

This exemplary test formulation was highly active and clear inhibition of *E. coli* growth was observed in broth tests conducted at 50 µl and 100 µl doses in 100 ml broth.

Table 9: *E. coli* 8739 Broth Test of Formulation (Optical Density at 590 nm)

Time Post Inoculation	Volume of Test Formulation (µl)					
	500	**100**	**50**	**10**	**5**	**Control**
0	0.958	0.063	0.019	0.007	0.007	0.003
1	0.708	0.025	0.028	0.024	0.021	0.022
2	0.762	0.023	0.053	0.081	0.087	0.102
3	0.547	0	0.094	0.158	0.179	0.191
4	0.486	0	0.140	0.270	0.286	0.323
5	0.594	0	0.181	0.316	0.311	0.345
6	0.522	0.011	0.238	0.361	0.367	0.401
6.5	0.579	0.014	0.262	0.376	0.372	0.411
23	0.617	0.031	0.285	0.619	0.747	0.654
24	0.553	0.058	0.286	0.606	0.740	0.683

The 16 hour old *E. coli* culture used as the inoculum had an OD at 590 nm of 0.697 units.

Further broth tests were conducted against two pathogenic strains of *E. coli* (700728, 12795) and an antimicrobial agent test strain at 50 µl and 100 µl.

Table 10: ODs at 16 hours of *E. coli* Cultures used as the Inoculum for the Multi-Strain Broth Test	
E.coli test strain	Optical density at 590 nm (absorbance units)
25922	0.852
700728	0.423
12795	0.395

1

2

3

Table 11: Results of a multi-strain Trial of Test formulation									
Time Post Inocul-ation (hours)	*E. coli* strain/Volume of Test formulation (µl)								
	25922			700728			12795		
	0	50	100	0	50	100	0	50	100
0	0.006	0.040	0.088	0.006	0.032	0.077	0.005	0.040	0.074
1	0.034	0.021	0.031	0.020	0.030	0.028	0.017	0.024	0.035
2	0.129	0.039	0.017	0.075	0.055	0.023	0.055	0.046	0.026
3	0.263	0.074	0.018	0.197	0.106	0.013	0.133	0.101	0.024
4	0.365	0.0138	0.011	0.335	0.188	0.019	0.311	0.198	0.044
5	0.366	0.174	0.004	0.312	0.240	0.050	0.364	0.205	0.049
6	0.395	0.234	0.009	0.371	0.264	0.044	0.399	0.264	0.038
7	0.427	0.256	0.009	0.406	0.287	0.040	0.436	0.282	0.042
23.5	0.688	0.351	0.049	0.545	0.323	0.096	0.564	0.296	0.079
24.5	0.683	0.349	0.054	0.561	0.323	0.106	0.582	0.279	0.083

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Table 9 summarises the results of the 50 and 100 µl/100 ml broth test. These results indicated good activity against *E. coli* 8739. Table 11 indicates that the test formulation showed good activity when challenged with other strains of *E. coli* including two pathogenic strains 700728, 12795.

The 100 µl dose of the test formulation had the lowest OD readings, therefore indicating greater inhibition of cell proliferation. 50 µl/100 ml broth of the test formulation appeared to have both slowed cell proliferation and reduced the final number of cells present in the broth. Where no test formulation was present, growth was rapid for all strains tested, especially in the first 4 hours after inoculation.

The test formulation is only one of a range of terpene formulations investigated so far and it is clearly very active. Clear inhibition of *E. coli* growth has been observed in broth tests conducted at 50 µl and 100 µl/100 ml broth, both against anti microbial assay strains and against pathogenic strains.

Formulations have been developed now which show very great activity against potentially lethal strain 0157: H7 of *E. coli*, both at very high innocula which are not sustainable in life and at levels which, though likely to be fatal, are found. Three 100 ml bottles were each filled with McConkey

broth to which was added one of either 20 µg/ml oxacillin, or 10 µg/ml of amoxicillin, or 1 µg/ml of the exemplary test terpene formulation. Each bottle was then inoculated with 10^4 *E. coli* 0157:H:7 and incubated for 24 hours at 35°C. Following incubation, the McConkey broth containing the oxacillin had lost its magenta colour and become yellowish and turbid, indicating that the antibiotic had been overwhelmed by the *E. coli*. The McConkey broth in the bottle containing the amoxicillin had only slightly reduced magenta colour, indicating that the antibiotic had contained the *E. coli*, whereas the McConkey broth in the bottle containing the terpene sample had an undiminished magenta colour.

This experiment was then repeated under the same conditions, except that the inoculum *of E. coli* 0157:H:7 was 10^8. In this case, both the oxacillin and amoxicillin samples were overwhelmed but the McConkey broth in the bottle containing the terpene sample had an undiminished magenta colour, indicating that, even with this extremely high inoculum, no growth had occurred.

Experiments have been carried out on xanthomonads including assay strains such as *Xylefa maltifolia* and plant pathogens such as *X. fastidiosa*. The latter causes Pierce's disease which has devastated grape culture in Southern California and threatens the wine growing areas of Napa Valley and Sonoma

Valley. The organisms are highly susceptible to terpene formulations according to the present invention.

It will be apparent for those skilled in the art that a number of modifications and variations may be made without departing from the scope of the present invention as set forth in the appending claims.

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CLAIMS

1. An antimicrobial composition for preventing or treating digestive tract infections, said composition comprising a terpene or a mixture of two or more terpenes.

2. The composition as claimed in Claim 1 which comprises a mixture of the terpenes carvone and geraniol.

3. The composition as claimed in either one of Claims 1 and 2 which further comprises a surfactant.

4. The composition of Claim 3 which consists of 1 to 99% terpenes and 1 to 99% surfactant.

5. The composition as claimed in either one of Claims 3 and 4 wherein the terpene or terpene mixture are natural or synthetic terpenes selected from citral, b-ionone, geraniol, carvacrol, eugenol, carvone, terpeniol, anethole or other generally recognized as safe terpenes with biocidal properties, and the surfactant is selected from polysorbate-80, polysorbate-20, polysorbate-40, polysorbate-60, polyglyceryl esters, polyglyceryl monooleate, decaglyceryl monocaprylate, propylene glycol dicaprilate, triglycerol monostearate or their combination.

6. The composition as claimed in any one of Claims 1 to 5 wherein the terpene or terpene mixture is at least partially encapsulated in a liposome to form a liposome-terpene(s) combination.

7. The composition as claimed in Claims 1 to 6 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is part of the inner core of a gelatin or cellulose capsule.

8. The composition as claimed in Claims 1 to 6 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is freeze dried, spray dried or dried in order to form a powder for encapsulation or solubilization.

9. The composition as claimed in Claims 1 to 6 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is freeze dried, spray dried or dried in order to be compressed in pill or tablet form.

10. The composition as claimed in Claims 1 to 6 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is freeze dried, spray dried or dried in order to be compressed in pill/tablet and coated for absorption in different areas along the gastro-intestinal tract.

11. A method to prevent or treat microbial infections of the digestive tract, said method

comprising orally administering a composition as claimed in any one of Claims 1 to 10 to patient.

12. The method as claimed in Claim 11 wherein the digestive tract infections are produced by normal, pathogenic or opportunistic microorganisms or its toxins selected from *Aerobacter sp., Aspergillus sp., Bacillus sp., Campylobacter sp., Candida sp., Clostridia sp., Enterobacteriaceae sp., Enterococcus sp., Escherichia sp., Haemophilus sp., Helicobacter sp Klebsiella sp., Lactobacillus sp., Listeria sp., Propionibacter sp., Pasteurella sp., Proteus sp., Pseudomonas sp., Salmonella sp., Shigella sp., Staphylococcus sp., Streptococcus sp. and Yersennia sp.*

13. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is effective against pathogenic and normal microflora comprising of *Aerobacter sp., Aspergillus sp., Bacillus sp., Campylobacter sp., Candida sp., Clostridia sp., Enterobacteriaceae sp., Enterococcus sp., Escherichia sp., Haemophilus sp., Helicobacter sp Klebsiella sp., Lactobacillus sp., Listeria sp., Propionibacter sp., Pasteurella sp., Proteus sp., Pseudomonas sp., Salmonella sp., Shigella sp., Staphylococcus sp., Streptococcus sp. and Yersennia sp.*

14. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is effective against pathogenic and opportunistic microorganisms causing traveler's diarrhea.

15. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is effective against pathogenic and opportunistic microorganisms causing ulcers along the digestive tract.

16. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is effective against anthrax.

17. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is effective against pathogenic and opportunistic microorganisms causing scours in calves.

18. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination is effective against pathogenic and opportunistic microorganisms causing scours in neonates and weaned piglets.

19. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination at lower concentrations has a bacteriostatic effect against pathogenic and normal gastro-intestinal microflora.

20. The method as claimed in Claim 11 wherein the terpene, terpene mixture or the liposome-terpene(s) combination at higher concentrations has a bactericidal effect against pathogenic and normal gastro-intestinal microflora.

21. The method as claimed in any one of Claims 11 to 20 wherein the effective dose of the terpene, the mixture of terpenes or the liposome-terpene(s) combination is between 20 mg and 5000 mg.

22. The method as claimed in any one of Claims 11 to 20 wherein the effective dose of the terpene, the mixture of terpenes or the liposome-terpene(s) combination is between 20 ppm and 50000 ppm in water and/or food consumed by the human or animal.

23. The method as claimed in any one of Claims 11 to 22 wherein the terpene, the mixture of terpenes or the liposome-terpene(s) combination is prepackaged in liquid form for oral consumption by humans or animals.

24. The method as claimed in any one of Claims 11 to 23 wherein the terpene, a mixture of terpenes or the liposome-terpene(s) combination is mixed with milk replacer and fed to calves and piglets.

25. The method as claimed in any one of Claims 11 to 24 wherein the terpene, the mixture of terpenes or the liposome-terpene(s) combination is intubated directly into the stomach of an animal.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/00015

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/11 A61K31/045 //(A61K31/11,31:045)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, CHEM ABS Data, EPO-Internal, PAJ, MEDLINE, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOYANOVA LUDMILA ET AL: "Inhibitory effect of rose oil products on Helicobacter pylori growth in vitro: Preliminary report." JOURNAL OF MEDICAL MICROBIOLOGY, vol. 48, no. 7, July 1999 (1999-07), pages 705-706, XP002196283 ISSN: 0022-2615 the whole document	1-5
Y	---	6-10
X	WO 97 02040 A (BEVILACQUA MARIA ;MICHELIN LAUSAROT ELISA (IT); BEVILACQUA MATTEO) 23 January 1997 (1997-01-23) claims 1-4 page 8, line 9-12 page 11, line 25-30	1,3-5, 7-10

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[X] Further documents are listed in the continuation of box C.

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16 April 2002	03/05/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Herrera, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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